1. TRIzol Homogenization

Homogenizing samples

1. Determine your sample type, and perform homogenization at room temperature according to the table below. The sample volume should not exceed 10% of the volume of TRIzol® Reagent used for homogenization. Be sure to use the indicated amount of TRIzol® Reagent, because an insufficient volume can result in DNA contamination of isolated RNA.

Sample Type	Action
Tissues	1. Add 1 mL TRIzol® Reagent per 50–100 mg of tissue sample. 2. Homogenize sample using a glass-Teflon® or power homogenizer. **Note**: Process or freeze tissue samples *immediately* upon collection.

3. Proceed to **Phase separation**, or store the homogenized sample. Homogenized samples can be stored at room temperature for several hours, or at –60 to –70°C for at least one month.

Phase separation

1. Incubate the homogenized sample (see **Homogenizing samples**) for 5 minutes at room temperature to permit complete dissociation of the nucleoprotein complex.
2. Add 0.2 mL of chloroform per 1 mL of TRIzol® Reagent used for homogenization. Cap the tube securely.
3. Shake tube vigorously by hand for 15 seconds.
4. Incubate for 2–3 minutes at room temperature.
5. Centrifuge the sample at 12,000 × *g* for 15 minutes at 4°C.

   Note: The mixture separates into a lower red phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. *RNA remains exclusively in the aqueous phase.* The upper aqueous phase is ~50% of the total volume.
6. Remove the aqueous phase of the sample by angling the tube at 45° and pipetting the solution out. Avoid drawing any of the interphase or organic layer into the pipette when removing the aqueous phase.

2. RNAqueous-4PCR Kit

- This kit assumes that you'll generate samples from tissue using their lysis/binding solution. We don't. Therefore, jump in at step 4 with the TRIzol lysate

See Figure 2 for estimation of mass values for small pieces of tissue. The recommended procedure for storage of tissue prior to RNA isolation is to put dissected tissue directly into 5 volumes of RNA*later* tissue collection/RNA stabilization solution (Ambion P/N AM7020). Store the tissue, immersed in RNA*later*, at 4°C for short-term storage (up to 1 week), or at –20°C for long-term storage.

Alternatively, fresh tissue can used provided it is processed immediately, or tissue can be snap-frozen in liquid nitrogen.

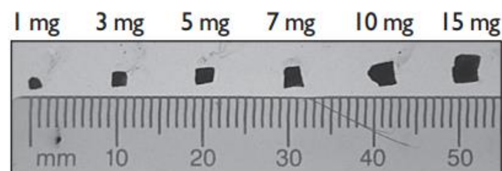


Figure 2. Estimating Mass of Small Tissue Samples

Pieces of mouse liver tissue weighing from 1–15 mg, were placed on a 1 mm scale ruler, and photographed. All pieces are approximately 1–2 mm thick. Most other soft tissues of are of similar density.

4. Draw the lysate/ethanol mixture through a Filter Cartridge

a. Apply the lysate/ethanol mixture (from the previous step) to a Filter Cartridge assembled in either a Collection Tube (supplied) or a 5 mL syringe barrel on a vacuum manifold.

The maximum volume that can be applied at one time is ~700 µL.

If you are using a vacuum manifold, apply the vacuum to draw the lysate through the filter. As soon as there is no more liquid resting on top of the filter, the remaining lysate can be applied. Once the entire sample has been passed through the filter, proceed to step 5. Do not release and reapply the vacuum between wash steps, simply leave it on until all of the wash steps are finished.

If the filter clogs, try inserting it into one of the Collection Tubes and centrifuging at RCF 10,000–15,000 x g until the mixture is through the filter.

b. Centrifuge at RCF 10,000–15,000 x g (typically 10,000–14,000 rpm) for ~15 sec–1 min or until the lysate/ethanol mixture is through the filter.

c. Discard the flow-through and reuse the Collection Tube for the washing steps.

d. Repeat as necessary with ~700 µL aliquots until all of the sample has been drawn though the filter. Generally up to ~2 mL of sample mixture can be passed through the filter without clogging or exceeding its RNA binding capacity.

5. Wash with 700 µL Wash Solution #1

Apply 700 µL Wash Solution #1 to the Filter Cartridge.

Draw the washes through the filter as in the previous step. Discard the flow-through and reuse the tube for subsequent washes.

6. Wash with 2 x 500 µL Wash Solution #2/3

a. Add 500 µL Wash Solution #2/3. Draw the wash solution through the filter as in the previous step.

b. Repeat with a second 500 µL aliquot of Wash Solution #2/3.

c. After discarding the wash solution, continue centrifugation, or leave on the vacuum manifold for ~10–30 seconds to remove the last traces of wash solution.

7. Elute RNA with 40–60 µL preheated Elution Solution

a. Put the Filter Cartridge into a fresh Collection Tube.

b. Pipet Elution Solution preheated to ~70–80°C to the center of the filter. Close the cap of the tube.
The exact volume of Elution Solution used is not critical. The amount of Elution Solution should correlate to the amount of RNA expected, in other words, RNA from samples close to the maximum size should be eluted with more Elution Solution than RNA from smaller samples. For maximum elution efficiency it is important to elute the RNA using 2 sequential applications of Elution Solution. The minimum practical volume of Elution Solution to use is 50 µL, applied as sequential aliquots of 40 µL and 10 µL.

c. Recover eluate by centrifugation for ~30 seconds at room temperature (RCF 10,000–15,000 x g).

8. Elute with a second 10–60 µL aliquot of Elution Solution

Add a second aliquot of hot Elution Solution to the center of the filter and re-spin for ~30 seconds.
Typically, this second elution is collected into the same tube as the first elution.

E. (optional) DNase 1 Treatment and DNase Inactivation

No RNA isolation procedure can guarantee the complete removal of trace amounts of DNA below the limit of detection by RT-PCR. DNase 1 treatment is recommended for RNA that will be used for RT-PCR because it effectively removes trace DNA contamination from RNA. It is especially important that no DNA is present in RT-PCRs using primers that do not flank introns, or for genes that have processed pseudogenes, because the RT-PCR products from RNA and contaminating DNA cannot be distinguished by size in these cases.

The DNase I must be removed from RNA that will be subjected to RT-PCR because it could degrade DNA made in the PCR. The DNase Inactivation Reagent also removes divalent cations introduced by the DNase 1 Buffer. This is important because divalent cations can degrade RNA at temperatures typically used for RNA denaturation prior to reverse transcription.

1. Add 0.1 volume of 10X DNase 1 Buffer and 1 µL of DNase 1

Mix gently.
If the RNA was eluted in >100 µL Elution Solution, use 1 µL DNase I per 100 µL RNA (in Elution Solution). If the RNA was eluted into ≤100 µL Elution Solution, use 1 µL DNase I per sample.

2. Incubate 15–30 min at 37°C

The RNA should now be free from contaminating DNA. The next few steps remove the DNase from the prep.

3. Add 0.1 volume DNase Inactivation Reagent

The DNase Inactivation Reagent is supplied as a slurry and must be dispersed by vortexing or pipetting prior to use.

The DNase Inactivation Reagent may become difficult to pipet after being used multiple times, due to depletion of fluid from the interstitial spaces. If this is the case, add a volume of nuclease-free water equal to about one-tenth the volume of the remaining Inactivation Reagent, and re-vortex to make a pipettable slurry.

4. Mix gently, incubate 2 min at RT

Flick the tube gently to disperse the DNase Inactivation Reagent in the reaction. Flick the tube once more during the 2 min incubation to re-disperse the reagent.

5. Centrifuge the tube at 10,000 x g for ~1 min to pellet the DNase Inactivation Reagent

In general it is not necessary to remove the RNA solution from the pelleted DNase Inactivation Reagent for short term storage, but avoid transferring any of the DNase Inactivation Reagent into subsequent reactions. For long term storage of RNA, we recommend removing the RNA to a new tube.

3. NanoDrop the RNA to determine appropriate concentrations for subsequent cDNA generation.

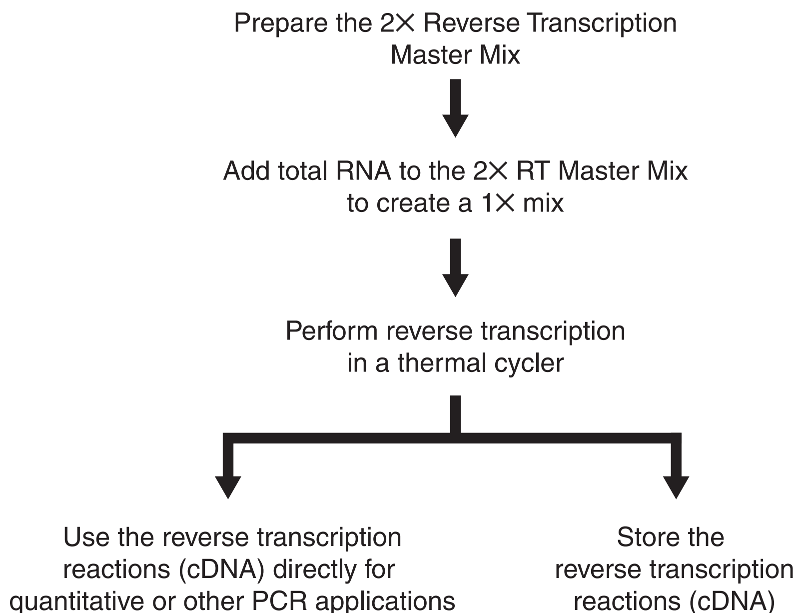
- Use Peter’s NanoDrop excel file for calculations.
- The file also contains mixture volumes for cDNA mix.

4. Generate cDNA using following protocol:

Using the High Capacity cDNA Reverse Transcription Kits

Overview

To synthesize single-stranded cDNA from total RNA using the High Capacity cDNA Reverse Transcription Kits:



RNA Template Guidelines

For optimal performance of the High Capacity cDNA Reverse Transcription Kits, Applied Biosystems recommends using RNA that is:

- Free of inhibitors of reverse transcription and PCR
- Dissolved in PCR-compatible buffer or water
- Free of RNase activity

Note: If you suspect that the RNA contains RNase activity, add RNase Inhibitor to the reverse transcription reaction at a final concentration of 1.0 U/µL.

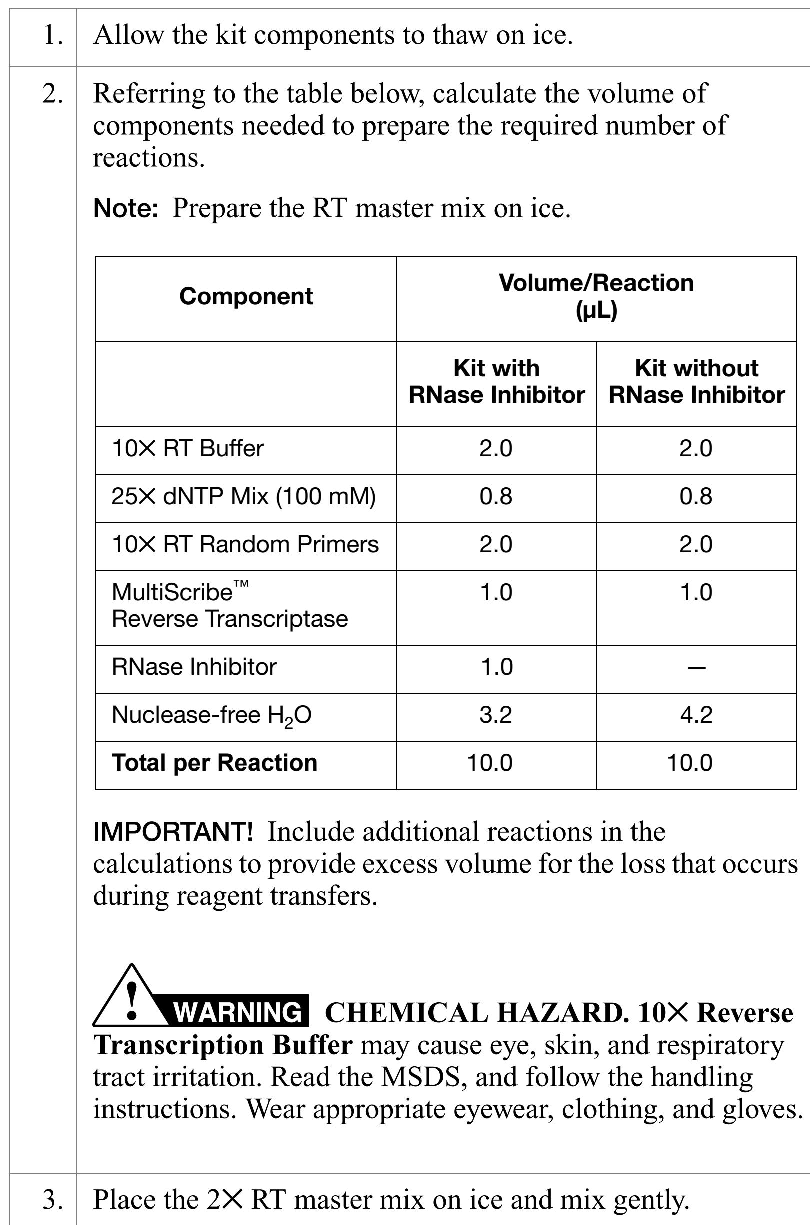
Input Amount of Total RNA

Use up to 2 µg of total RNA per 20-µL reaction.

Preparing the 2✕ Reverse Transcription Master Mix

Prepare the 2✕ RT master mix using the kit components before preparing the reaction plate.

To prepare the 2✕ RT master mix (per 20-µL reaction):



1. Allow the kit components to thaw on ice.
2. Referring to the table below, calculate the volume of components needed to prepare the required number of reactions.

   Note: Prepare the RT master mix on ice.

Component	Volume/Reaction (µL)	
	Kit with RNase Inhibitor	Kit without RNase Inhibitor
10✕ RT Buffer	2.0	2.0
25✕ dNTP Mix (100 mM)	0.8	0.8
10✕ RT Random Primers	2.0	2.0
MultiScribe™ Reverse Transcriptase	1.0	1.0
RNase Inhibitor	1.0	—
Nuclease-free H_2O	3.2	4.2
Total per Reaction	10.0	10.0

   IMPORTANT! Include additional reactions in the calculations to provide excess volume for the loss that occurs during reagent transfers.

   WARNING **CHEMICAL HAZARD. 10✕ Reverse Transcription Buffer** may cause eye, skin, and respiratory tract irritation. Read the MSDS, and follow the handling instructions. Wear appropriate eyewear, clothing, and gloves.

3. Place the 2✕ RT master mix on ice and mix gently.

Preparing the cDNA Reverse Transcription Reactions

To prepare the cDNA RT reactions:

1.	Pipette 10 µL of 2✕ RT master mix into each well of a 96-well reaction plate or individual tube.
2.	Pipette 10 µL of RNA sample into each well, pipetting up and down two times to mix.
3.	Seal the plates or tubes.
4.	Briefly centrifuge the plate or tubes to spin down the contents and to eliminate any air bubbles.
5.	Place the plate or tubes on ice until you are ready to load the thermal cycler.

Performing Reverse Transcription

To perform reverse transcription:

1. Program the thermal cycler conditions using one of the thermal cyclers listed in Table 3 on page 4.

   IMPORTANT! These conditions are optimized for use with the High Capacity cDNA Reverse Transcription Kits.

	Step 1	Step 2	Step 3	Step 4
Temperature (°C)	25	37	85	4
Time	10 min	120 min	5 min	∞

2. Set the reaction volume to **20 µL**.
3. Load the reactions into the thermal cycler.
4. Start the reverse transcription run.

Storing cDNA Reverse Transcription Reactions

You can store cDNA RT plates or tubes prepared using the High Capacity cDNA Reverse Transcription Kits for short-term or long-term storage.

Storage Duration	Storage Temperature (°C)
Short-term (up to 24 hours before use)‡	2 to 6
Long-term	−15 to −25

‡ For prolonged storage at 2 to 6 °C, add EDTA to a final concentration of 1 mM to chelate cations and to prevent nucleic acid degradation.

IMPORTANT! If required, briefly centrifuge the archive plates or tubes before storing to spin down the contents and to eliminate any air bubbles.

5. Run PCR using cDNA as template.

- Use Peter’s qPCR excel file to organize plates/tubes and to calculate reaction volumes.

6. Run PCR reactions in Doug’s thermocycler.

- Don’t forget to sign up for it.